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In vitro antibacterial activities of Broccoli (*Brassica oleracea* L.var *italica*) against food borne bacteria

Sibi G[1*], Abhilasha Shukla[1], K. Dhananjaya[1], K.R. Ravikumar[1] and H. Mallesha[2]
[1]R&D Centre, Robust Materials Technology Pvt. Ltd. Bengaluru, Karnataka, India
[2]R&D Centre, Robust Herbals Pvt. Ltd. Bengaluru, Karnataka, India

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ABSTRACT

Foodborne diseases remain substantial and safety of food is an important health, social and economical issue. Food borne illnesses caused by microbial contamination raises concerns to find alternate sources which are safe to human and environmental health. This study was investigated to determine the antibacterial activity of broccoli (*Brassica oleracea* L. var. *italica*). Various solvent extracts of broccoli were prepared and analyzed for their phytoconstituents. A total of six food borne bacteria viz., *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* MTCC 3224 and *Shigella flexneri* ATCC 12022 were tested against the broccoli extracts. Minimum inhibitory concentration (MIC) values of 10 - 320 µg ml^{-1} were recorded against most of the pathogens with acetone and methanol as the potential extracts. B. subtilis ATCC 6633 (15.4 mm) and Bacillus cereus ATCC 10876 (16.3 mm) were found to be the most sensitive organisms among the pathogens tested.

INTRODUCTION

Food borne diseases are resulting from consumption of contaminated food with pathogenic bacteria and their metabolites. Identification and evaluation of effective antibacterial agents from natural products for controlling food borne pathogens and assure safe food supply is a global concern. Use of synthetic food preservatives may lead to negative health consequences. Food additives such as monosodium glutamate, aspartame, saccharin, sodium cyclamate, sulfites, nitrates, nitrites and antibiotics causes headache, nausea, weakness, mental retardation, seizures, cancer and anorexia (Rangan and Barceloux, 2009, Wroblewska, 2009). Increasing concern about the toxic chemical preservatives, demand for food with longer shelf life with no or less chemical preservatives put pressure to find alternatives for better healthcare. Naturally occurring bioactive compounds from plant origin have greater antimicrobial activity than purified constituents (Delaquis *et al.,* 2002). *Brassicaceae* (=*Cruciferae*) vegetables represent an important part of the human diet worldwide and are considered important food crops in India, China, Japan and European countries.

** Corresponding Author*
Dr. Sibi G, R and D Centre, Robust Materials Technology Pvt. Ltd. Bengaluru - 560072, Karnataka, India. Telephone : +91 80 2318 6230

Brassicaceae vegetables have been reported to have anticancer and antioxidant properties (Keck and Finley, 2004). Broccoli (*Brassica oleracea* L. var. *italica*) belongs to the family Brassicaceae having leaves which are more divided and petiolate. The main head consists of clusters of fully differentiated flower buds which are less densely arranged with longer peduncles. Sprouting forms of broccoli bear many small flowers heads. It is an annual herb reaching 400 mm during vegetative stage and 1-2 m at the end of flowering (Dixon, 2007). Broccoli has antimicrobial (Survay *et al.,* 2012, Jaiswal *et al.,* 2011), antioxidant (Mahn and Reyes, 2011) and anticancer (Vasanthi *et al.,* 2009, Moreno *et al.,* 2006, Fahey *et al.,* 1997) activities. This study has evaluated the antimicrobial potential of broccoli extracts against food borne bacteria with a view to exploring its potential application in food industries as botanical preservatives.

MATERIAL AND METHODS

Preparation of extracts

Broccoli vegetables were purchased from local market and the florets were removed from head, dried, pulverized and extracted with solvents of increasing polarity (petroleum ether, chloroform, ethyl acetate, acetone, methanol and aqueous) at room temperature for 48 hrs.

The extracts were filtered using Whatman No.1 filter paper and concentrated to dryness under reduced pressure in a rotary evaporator and stored in sterile vials at 4°C until used.

Test organisms

Bacillus cereus ATCC 10876, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* MTCC 3224 and *Shigella flexneri* ATCC 12022 were used in this study. The bacteria were then standardized by adjusting the bacterial suspension to absorbance reading within the range of 0.08 to 0.10 at OD 625 nm which was equivalent to 1-2 × 10^8 CFU/mL.

Phytochemical analysis

Phytochemical analysis of the solvent extracts of broccoli was performed by following standard procedures (Trease and Evans, 2002; Harborne, 1998 and Sofowara, 1993). In brief, 0.5 ml of extract was added with a drop or two of Mayer's regent by the side of test tube and the formation of white or creamy precipitate indicates presence of alkaloids. Adding 1 ml of extract with ammonia and conc. sulphuric acid and disappearance of yellow colour on standing indicates flavonoids. Formation of brown ring at interface by the addition 2 ml of glacial acetic acid followed by few drops of ferric chloride solution and 1 ml of conc. sulphuric acid to the extracts revealed the presence of glycosides. Adding few drops of neutral ferric chloride to the extract and development of dark green color indicates the presence of the phenolic compounds. Existence of froth formation during warming and vigorous shaking indicates saponins. Change of colour from violet to blue or green after the addition of 2 ml of acetic anhydride and sulphuric acid gives positive result for steroids. Appearance of brownish green or blue black coloration after adding 0.1% ferric chloride to the cooled extract indicates tannins. Addition of 2 ml of chloroform and 3 ml of conc. sulphuric acid to the extract and formation of reddish brown layer at the junction of two solutions confirms terpenoids.

Antibacterial assay

Preliminary antibacterial activity of various solvent extracts of broccoli was evaluated using well diffusion assay. 100µl of the appropriate bacterial suspension was inoculated on Mueller Hinton agar using sterile swabs. 20 µl of the extract was added into the 5 mm wells and the plates were allowed for pre-diffusion of the extract before incubation. The diameter of zone of inhibition mean of two replicates ± SD as indicated by clear area which was devoid of growth of microbes was measured to determine antibacterial activity.

Minimum Inhibitory concentration (MIC) Assay

Well diffusion method

The extracts were further evaluated for the determination of MIC by well diffusion technique and the concentrations used were 320, 160, 80, 40, 20 and 10 µg ml^{-1}. The zone diameter of inhibition was determined for the different concentrations tested. The data obtained were statistically analyzed and the results were expressed as means along with standard deviation of three parallel measurements.

Broth dilution method

MIC of spice extracts was tested by two fold dilution method. In brief, the extracts were dissolved in respective solvents and added into Luria-Bertani (LB) broth to obtain a concentration of 640 µg/ml and serially diluted to achieve 320, 160, 80, 40, 20 and 10 µg/ml. A 10 µl standardized suspension of each tested organism (10^7 CFU/ml) was transferred to each tube. The control tubes containing only bacterial suspension were incubated at 37°C for 24 hrs. The lowest concentration of the extract which did not show any growth of tested organism was determined as the MIC.

RESULT AND DISCUSSION

Phytochemical analysis revealed the presence of flavonoids, glycosides, saponins, steroids, terpenoids and alkaloids (table-1). The data pertaining to antibacterial potential of various solvent extracts of broccoli are represented in table-2. Preliminary antibacterial studies of broccoli extracts demonstrated its broad activity against the food borne pathogens. Most of the extracts have exhibited antibacterial activity with zone diameter range of 0.1 - 15.4 mm. B. subtilis ATCC 6633 (0.6-15.4 mm) and Bacillus cereus ATCC 10876 (8.9-16.3 mm) were found to be the most sensitive organisms in the study. Aqueous extract was completely failed to control the growth of pathogens tested.

Table. 1: Phytochemical analysis of Broccoli extracts.

Test	Pet	Chl	Eta	Ace	Met	Aqu
Alkaloids	-	-	-	+	-	+
Flavonoids	+	-	+	+	+	-
Glycosides	-	+	+	-	+	-
Phenols	-	-	-	-	-	-
Saponins	+	-	-	-	+	+
Steroids	-	-	+	+	+	-
Tannins	-	-	-	-	-	-
Terpenoids	+	-	-	-	+	+

(+) presence (-) absence

Pet-petroleum ether, Chl-chloroform; Eta-ethyl acetate; Ace-acetone; Met-methanol; Aqu-aqueous

Minimum inhibitory concentrations (MIC) of the various solvent extracts of broccoli performed in well diffusion method revealed that acetone and methanolic extracts were significantly effective against most of the pathogens tested (table-3 and 4). However, highest zone diameter of inhibition (17.14 mm) was recorded with petroleum ether extract against *B.subtilis*. Chloroform extract was effective against *B.cereus* (10.24 mm) and *S.typhi* (9.21 mm).

Moderate inhibitory activity of ethyl acetate extract was observed against *B.subtilis* (6.58 mm). Acetone and methanol extracts were effective against *B.cereus, B.subtilis, E.coli* and *S.flexneri.* MIC values obtained from broth dilution assay were in the range of ≤ 10 to ≥ 320 µg ml^{-1}(table-5). Lowest MIC was observed with acetone and methanol extracts against *B.cereus* and *B.subtilis* (≤ 10 µg ml^{-1}).

Table. 2: Antibacterial Assay of crude broccoli extract.

Organism	Zone diameter of Inhibition (mm)					
	Pet	Chl	Eta	Ace	Met	Aqu
B. cereus ATCC 10876	NA	16.3 ± 1.5	NA	12.0 ± 0.7	8.9 ± 0.1	NA
B. subtilis ATCC 6633	15.4 ± 0.3	0.6 ± 0.1	3.2 ± 0.9	4.1 ± 0.2	9.7 ± 0.87	NA
S. aureus ATCC 6538	11.9 ± 0.7	NA	6.1 ± 0.0	NA	NA	NA
E .coli ATCC 8739	NA	NA	NA	13.8 ± 1.8	6.7 ± 0.4	NA
S. typhi MTCC 3224	NA	11.7 ± 0.4	NA	1.6 ± 0.7	NA	NA
S. flexneri ATCC 12022	NA	NA	NA	15.1 ± 0.5	11.8 ± 0.0	NA

Note: NA - no activity

Table. 3: Zone diameter of inhibition (mm) in MIC by well diffusion assay (µg/ml) against Gram positive bacteria.

	***B. cereus* ATCC 10876**						***B. subtilis* ATCC 6633**						***S. aureus* ATCC 6538**					
	10	**20**	**40**	**80**	**160**	**320**	**10**	**20**	**40**	**80**	**160**	**320**	**10**	**20**	**40**	**80**	**160**	**320**
Pet	-	-	-	-	-	-	16.01	16.21	17.14	16.07	17.08	NG	-	-	-	5.04	7.85	9.64
Chl	-	-	-	9.33	9.67	10.24	-	-	-	-	-	-	-	-	-	-	-	-
Eta	-	-	-	-	-	-	-	-	-	3.10	5.31	6.58	-	-	-	-	-	4.69
Ace	11.12	11.64	12.20	12.23	13.09	15.61	-	-	-	3.86	3.03	7.01	-	-	-	-	-	-
Met	-	4.18	6.05	6.35	8.34	11.87	6.14	6.98	7.28	7.37	8.26	11.00	-	-	-	-	-	-
Aqu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: NG - no growth

Table. 4: Zone diameter of inhibition (mm) in MIC by well diffusion assay (µg/ml) against Gram negative bacteria.

	***E. coli* ATCC 8739**						***S. typhi* MTCC 3224**						***S. flexneri* ATCC 12022**					
	10	**20**	**40**	**80**	**160**	**320**	**10**	**20**	**40**	**80**	**160**	**320**	**10**	**20**	**40**	**80**	**160**	**320**
Pet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chl	-	-	-	-	-	-	6.12	6.20	7.41	6.34	7.63	9.21	-	-	-	-	-	-
Eta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ace	3.00	3.96	6.74	7.04	9.34	18.1	-	-	-	-	-	3.94	4.14	4.52	5.38	5.63	8.11	10.01
Met	2.36	4.08	6.11	6.96	7.57	10.35	-	-	-	-	-	-	5.00	5.09	5.64	6.67	8.34	10.14
Aqu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table. 5: Minimum inhibitory concentrations in broth dilution assay.

Ext	Minimum Inhibitory Concentration (µg/ml)					
	***B. cereus* ATCC 10876**	***B. subtilis* ATCC 6633**	***S. aureus* ATCC 6538**	***E. coli* ATCC 8739**	***S. typhi* MTCC 3224**	***S. flexneri* ATCC 12022**
Pet	-	≤ 10	≥ 160	-	-	-
Chl	≤ 160	-	-	-	≤ 40	-
Eta	-	-	≥ 320	-	-	-
Ace	≤ 10	320	-	80	≥ 320	160
Met	≤ 160	40	-	≤ 160	-	≤ 160
Aqu	-	-	-	-	-	-

S.aureus ATCC 6538 was sensitive to petroleum ether and ethyl acetate extracts with the MIC values of ≥ 160 and ≥ 320 µg ml^{-1} but other extracts had no activity against the food borne pathogen. Previous studies recorded the inhibitory activity of broccoli derived compound against methicillin resistant *S.aureus* (Johansson *et al.,* 2008). In this study, *S.typhi* MTCC 3224 was inhibited with a MIC ranging from 160 - 320 µg ml^{-1} and antibacterial activity with MIC of 625 - 1250 µg ml^{-1} against *S.typhimurium* was determined in earlier findings (Survay *et al.,* 2012). Farzinebrahimi *et al.,* (2012) has reported the antibacterial activity of leaf extracts of broccoli against *Pseudomonas aeroginosa*. Antimicrobial activity of *Brassica oleracea* varieties were reported by previous studies (Brandi *et al.,* 2006, Sousa *et al.,* 2008) and in this study, inhibitory activity of broccoli has been revealed against food borne pathogenic bacteria.

CONCLUSION

From the results, it would seem logical to predict that consumption of broccoli could prevent the ailments caused by food-borne pathogens. Further, owing to its strong antibacterial activity, bioactive compounds from broccoli (*Brassica oleracea* L. var. *italica*) have scope for the possible use in food industries to stay away from food borne pathogens.

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